Cell Host & Microbe
Article

# Memory CD8$^+$ T Cells Can Outsource IFN-$\gamma$ Production but Not Cytolytic Killing for Antiviral Protection

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http://dx.doi.org/10.1016/j.chom.2013.04.004

## SUMMARY

**Immunization with vaccinia virus (VACV), the virus comprising the smallpox vaccine, induces memory CD8$^+$ T cells that protect from subsequent infections with smallpox in humans or the related ectromelia virus (ECTV) in mice. Memory CD8$^+$ T cells largely mediate these effects by expanding into secondary effectors that secrete the antiviral cytokine interferon-$\gamma$ (IFN-$\gamma$) and induce cytolysis via releasing factors such as perforin, which permeabilizes target cells. We show that protection from ECTV infection after VACV immunization depends on the initial memory cell frequency and ability of expanded secondary effectors to kill infected targets in a perforin-dependent manner. Although IFN-$\gamma$ is essential for antiviral protection, it can be produced by either secondary effectors or concomitant primary effector CD8$^+$ T cells recruited to the response. Thus, during lethal virus challenge, memory CD8$^+$ T cells are required for cytolytic killing of infected cells, but primary effectors can play important roles by producing IFN-$\gamma$.**

## INTRODUCTION

The severity of a viral infection, from asymptomatic to lethal, depends on the balance between the swiftness and strength of the innate and adaptive immune responses and the speed of virus replication and spread in the permissive host. Vaccination expands the pool of antiviral lymphocytes and/or generates circulating antibodies altering this balance in favor of the host. This paradigm becomes vivid after footpad infection of different mouse strains with the Orthopoxvirus (OPV) ectromelia virus (ECTV). ECTV is a natural mouse pathogen that causes a disease known as mousepox. It is genetically and antigenically very similar to the virus of human smallpox and also to the virus in the smallpox vaccine, vaccinia virus (VACV) (Fenner et al., 1988). After footpad infection of all laboratory mouse strains, ECTV spreads lymphohematogenously (LHY) to seed the visceral organs, mainly the liver and spleen. However, the outcome of the infection varies depending on the mouse strain. C57BL/6 (B6) mice mount an effective innate natural killer cell (NKC) response in the draining lymph node (D-LN) at 2 days postinfection (dpi) followed by an adaptive CD8$^+$ T cell response that peaks in the D-LNs at 5 dpi and in the liver and spleen at 7 dpi (Fang et al., 2008, 2011; Fang and Sigal, 2005, 2006; Parker et al., 2007). As a consequence, B6 mice suffer a relatively mild infection without major clinical symptoms of disease. On the other hand, mice of the strains BALB/c, A/J, DBA/2J, and B6 congenic B6.D2-(D6Mit149-D6Mit15)/LusJ (B6.D2-D6) (Davis et al., 2005; Fang et al., 2011) generally succumb at 7–10 dpi, most likely due to the high virus titers and consequential massive necrosis of the liver (Wallace et al., 1985). In the case of the DBA/2J strain, a susceptibility gene has been mapped to the distal region of chromosome 6. This region is known as the NK complex (Delano and Brownstein, 1995) because it houses many NKC receptor genes, including *Klrd1*, which encodes CD94 and is not expressed in DBA2/J mice (Vance et al., 2002). Notably, *Klrd1*$^{-/-}$ B6 mice and B6.D2-D6 mice, which are congenic B6 mice with the NKC complex from DBA2/J mice, are susceptible to mousepox and both can be rescued by transgenic expression of CD94 (Fang et al., 2011).

As with humans and smallpox, susceptible mice can be protected from mousepox by immunization with VACV (Fenner, 1994). Thus, ECTV can be used as an invaluable experimental model to understand how the smallpox vaccine protects. In addition to its importance as a smallpox model, ECTV is a textbook model for the many human and animal viruses that become systemic and cause disease by disseminating LHY (Flint et al., 2009). Hence, ECTV is uniquely suited to study the mechanisms of acquired protection against the many viruses that spread using this route.

CD8$^+$ T cells play a major role in antiviral immunity. Antiviral CD8$^+$ T cells become effectors and proliferate when their T cell receptor specifically recognizes viral peptide determinants bound to major histocompatibility class I molecules (MHC I) at the surface of antigen presenting cells. Effector CD8$^+$ T cells contribute to reduce the severity of disease by killing infected cells and by producing antiviral cytokines (Harty et al., 2000). The major antiviral cytokine produced by effector CD8$^+$ T cells is interferon-$\gamma$ (IFN-$\gamma$) and the main cytolytic mechanism of

effector CD8+ T cells is granule exocytosis whereby they release proapoptotic enzymes, prominently granzyme B (GzB), into the cytosol of the target cell through pores formed by perforin (Prf) (Trapani and Smyth, 2002). However, IFN-γ production and granule exocytosis mediated-killing are hallmarks but not the exclusive domain of CD8+ T cells. NKCs, which recognize targets through germline encoded receptors, use these exact same mechanisms to control viruses during the early stages of infection (Cerwenka and Lanier, 2001). Moreover, IFN-γ production and granule exocytosis-mediated killing are also functions of NK T cells and CD4+ T cells (Billiau and Matthys, 2009; Fang et al., 2012; Marshall and Swain, 2011; Tupin et al., 2007).

If a virus is eliminated, most of the effector CD8+ T cells die, but an expanded pool of virus-specific CD8+ T cells remains. These memory CD8+ T cells coexist with a naive pool of antiviral CD8+ T cells that may have been present during the primary infection but did not encounter antigen or may be newly produced (Martin et al., 2011). When a subsequent infection with a pathogen that carries the appropriate viral peptide determinants occurs, the memory CD8+ T cells rapidly become secondary effectors and expand. Similar to the primary effectors, these secondary effectors can kill infected cells and produce IFN-γ, diminishing the seriousness of the infection (Welsh et al., 2004). Of note, by reducing virus loads, killing antigen-presenting cells, and/or advantageously competing for access to them, memory CD8+ T cells can also dampen the response of the coexisting antiviral naive cells (Guarda et al., 2007; Kedl et al., 2000). Yet, concomitant primary and secondary responses can occur, as demonstrated in other infection models (Badovinac et al., 2003; Martin et al., 2012; Martin et al., 2011; Turner et al., 2001). However, whether these concomitant primary responses can help combat the infection is unknown.

After primary infection, mousepox-susceptible mice, such as *Klrd1*$^{-/-}$ B6 mice and B6.D2-D6 mice, fail to mount CD8+ T cell responses (Fang et al., 2006; Fang et al., 2011). However, they mount strong CD8+ T cell responses to VACV. Due to their genetic similarity, most CD8+ T cell determinants from VACV and ECTV are identical (Remakus et al., 2012; Tscharke et al., 2005, 2006). As a consequence, memory CD8+ T cells from VACV-immune mice protect susceptible BALB/c mice from mousepox (Xu et al., 2007). Hence, the pairing of VACV immunization and challenge with ECTV serves as a unique model to study how memory CD8+ T cells protect from a lethal disease that spreads LHY within the context of a highly successful human vaccine. Using this model, we have previously shown that a major mechanism whereby memory CD8+ T cells protect from mousepox is by curbing lymphohematogenous (LH) spread (Xu et al., 2007). They do this by rapidly becoming secondary effectors and proliferating in the D-LN. However, given that other cells have similar functions, it is still unknown whether for this or any other infection protection from disease requires the secondary effectors to kill infected cells and also to produce IFN-γ. Moreover, it is also unknown whether concomitant primary and secondary responses can occur during infections with highly virulent viruses that normally curtail the primary CD8+ T cell response of the naive host. If so, it is possible that the concomitant primary response could play a role in protection, particularly if the secondary effectors are suboptimal.

Here we demonstrate that in the absence of other sources, the IFN-γ produced by secondary effectors is necessary and sufficient for protection from mousepox. However, when other sources of IFN-γ are available, the secondary effectors need to be capable of granule exocytosis-mediated killing but not IFN-γ production because this function can be complemented by a concomitant primary response. Our findings help understand the mechanisms whereby memory CD8+ T cells protect from viral diseases and unveil a hitherto unknown role for concomitant primary CD8+ T cell responses during the course of a secondary CD8+ T cell response.

## RESULTS

### In the Absence of Other Sources, IFN-γ Produced by Memory CD8+ T Cells Is Necessary and Sufficient for Protection from Mousepox

To investigate the need for IFN-γ during protection by memory CD8+ T cells, we took advantage of the fact that IFN-γ$^{-/-}$ mice (B6.129S7-Ifng$^{tm1Ts}$/J, IFN-γ$^{-/-}$) survive VACV infection and maintain a large memory CD8+ T cell pool that is highly cross-reactive with ECTV (Remakus et al., 2012; Remakus and Sigal, 2011). We transferred 5 × 10$^6$ purified CD8+ T cells obtained from naive wild-type (WT) B6 (N-WT) mice or VACV-immune WT B6 (M-WT) or IFN-γ$^{-/-}$ B6 (M-IFN-γ$^{-/-}$) mice into naive IFN-γ$^{-/-}$ mice. One day after transfer, the mice were challenged with ECTV. All mice that were recipients of N-WT cells succumbed, while all those that received M-WT survived the infection (Figure 1A) with minimal weight loss (Figure 1B). On the other hand, most M-IFN-γ$^{-/-}$ recipients succumbed (Figure 1A), and the few that survived became very sick as indicated by major weight loss (Figure 1B). Consistent with the survival data, the virus loads in the livers and spleens of M-WT recipients were several orders of magnitude lower than in those that received M-IFN-γ$^{-/-}$ or N-WT CD8+ T cells (Figures 1C and 1D). Histological analysis at 7 dpi showed that the livers of IFN-γ$^{-/-}$ mice that were recipients of N-WT or M-IFN-γ$^{-/-}$ CD8+ T cells were necrotic, were devoid of marked mononuclear cell infiltrates localized in the lesions, had extensive hepatocellular necrosis, and had a large number of foci that stained with rabbit antisera to the structural ECTV protein EVM-135. In contrast, the livers of IFN-γ$^{-/-}$ mice that received M-WT CD8+ T cells had very few necrotic areas, low tissue damage, a high number of infiltrating lymphocytes in lesions, and significantly less EVM135+ foci than those that received N-WT or M-IFN-γ$^{-/-}$ CD8+ T cells (Figure S1 available online). We also analyzed the CD8+ T cell responses at 7 dpi in the livers and spleens of IFN-γ$^{-/-}$ mice that were recipients of N-WT, M-WT and M-IFN-γ$^{-/-}$ CD8+ T cells. Liver-infiltrating mononuclear cell (LIMC) counts in the livers were comparable in all infected IFN-γ$^{-/-}$ mice and significantly higher than in uninfected mice (Figure 2A; representative flow cytometry plots in Figure S2). Splenocyte counts (Figure 2G) were higher in M-WT and M-IFN-γ$^{-/-}$ recipients than in uninfected mice (because some N-WT recipients succumbed before the analysis, we were unable to statistically compare them with the other groups for these parameters). However, in both liver and spleen, the absolute number of total CD8+ T cells (Figures 2B and 2H), total CD8+ T cells that constitutively expressed GzB (Figures 2C and 2I),

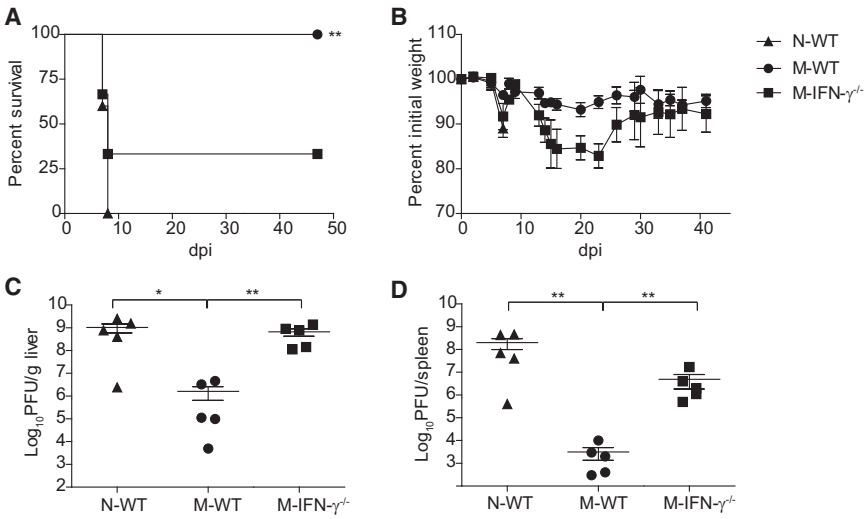



**Figure 1. M-WT but Not M-IFN-$\gamma^{-/-}$ CD8$^+$ T Cells Efficiently Protect IFN-$\gamma^{-/-}$ Mice from Mousepox**

(A) IFN-$\gamma^{-/-}$ mice received 5 × $10^6$ N-WT, M-WT, or M-IFN-$\gamma^{-/-}$ CD8$^+$ T cells and were infected with ECTV. Survival was monitored. The experiment is representative of three, where n = 5 for every group except for M-IFN-$\gamma^{-/-}$, where n = 6.

(B) The mice in (A) were weighed daily.

(C) IFN-$\gamma^{-/-}$ mice that received 5 × $10^6$ N-WT, M-WT, or M-IFN-$\gamma^{-/-}$ CD8$^+$ T cells were infected with ECTV. Seven days postinfection, mice were killed and virus titers were determined in liver. Data correspond to five mice per group ± SEM and are representative of two independent experiments.

(D) As in (C), but the virus titers were determined in spleen.

See also Figure S1 for liver pathology.

and total CD8$^+$ T cells specific for the K$^b$-restricted VACV/ECTV immunodominant determinant TSYKFESV$^+$ as detected with K$^b$-TSYKFESV dimers (Dimer X, BD) (Figure 2D and J) were significantly higher in M-WT and M-IFN-$\gamma^{-/-}$ recipients than in N-WT recipients (note that GzB expression does not require ex vivo restimulation during acute ECTV infection and serves as a marker of total antiviral effector CD8$^+$ T cells [Fang and Sigal, 2005]). Also, after ex vivo restimulation with TSYKFESV, there was significantly more CD107a-positive LIMCs and splenic CD8$^+$ T cells from M-WT and M-IFN-$\gamma^{-/-}$ recipients than from N-WT recipients (Figures 2E and 2K), which is a marker of cytotoxic CD8$^+$ T cell degranulation (Betts et al., 2003). As expected, only those cells from M-WT recipients produced IFN-γ (Figures 2F and 2L). These experiments demonstrate that the presence of IFN-γ is essential during protection by memory CD8$^+$ T cells and that the memory CD8$^+$ T cells can produce all the IFN-γ required for protection. These experiments also show that IFN-γ-deficient memory cells respond but do not protect in an IFN-γ-deficient environment.

## IFN-$\gamma^{-/-}$ but Not Prf$^{-/-}$ Memory CD8$^+$ T Cells Protect Susceptible IFN-$\gamma^+$ Mice from Lethal Mousepox

Given that M-IFN-$\gamma^{-/-}$ cells did not protect IFN-$\gamma^{-/-}$ mice but were able to respond to ECTV, we next tested whether they could protect mousepox-susceptible IFN-γ-sufficient B6.D2-D6 mice. Because Prf is another major CD8$^+$ T cell effector molecule required for granule exocytosis-mediated killing, we also tested whether memory CD8$^+$ T cells obtained from Prf-deficient (M-Prf$^{-/-}$) mice could protect from lethal mousepox. Graded numbers of N-WT, M-WT, M-IFN-$\gamma^{-/-}$, or M-Prf$^{-/-}$ CD8$^+$ T cells were adoptively transferred into B6.D2-D6 mice. The proportion of K$^b$-TSYKFESV-specific cells in the transferred populations was determined by flow cytometry (Figure 3A) and was used to calculate the approximate number of K$^b$-TSYKFESV-specific cells transferred. Upon ECTV challenge, all B6.D2-D6 mice that received N-WT or M-Prf$^{-/-}$ cells died, although the death of the M-Prf$^{-/-}$ recipients was slightly but significantly delayed (Figures 3B and 3C). All the mice that received ~60,000 or more K$^b$-TSYKFESV-specific M-WT or M-IFN-$\gamma^{-/-}$ CD8$^+$ T cells were significantly protected from death and did not show symptoms of disease except for relatively minor weight loss. Protection by the memory cells was dose dependent because all of the mice succumbed when adoptively transferred with only ~25,000 K$^b$-TSYKFESV-specific M-WT CD8$^+$ T cells (data not shown). Virus loads in liver (Figure 3D) and spleen (Figure 3E) at 7 dpi were significantly lower in M-WT and M-IFN-$\gamma^{-/-}$ than in M-Prf$^{-/-}$ and N-WT B6.D2-D6 recipients. While there was a tendency for higher virus loads in mice recipient of M-IFN-$\gamma^{-/-}$ than in M-WT, it was not statistically significant. Compared to those in N-WT recipients, the virus titers in the liver of M-Prf$^{-/-}$ recipients were moderately but significantly decreased indicating a low level of protection, which could explain their delayed death. Thus, contrary to what occurs with IFN-$\gamma^{-/-}$ mice, M-IFN-$\gamma^{-/-}$ cells can protect susceptible B6.D2-D6 mice from lethal mousepox. This suggests that the memory CD8$^+$ T cells can outsource the necessary IFN-γ production to other cells to protect from lethal mousepox. On the other hand, the expression of Prf cannot be outsourced.

## Endogenous Prf but Not IFN-γ in Memory CD8$^+$ T Cells Is Required for the Early Control of ECTV LH Spread

We have previously shown in BALB/c mice that that memory CD8$^+$ T cells curb LH spread to the liver and spleen. Similarly, at 4 dpi, the virus loads in the liver (Figure 4A) and spleen (Figure 4B) of M-WT and M-IFN-$\gamma^{-/-}$ B6.D2-D6 recipients were significantly lower than in N-WT and M-Prf$^{-/-}$ B6.D2-D6 recipients. While there was some significant protection of the spleen in M-Prf$^{-/-}$ recipients, it was much less pronounced than in M-WT or M-IFN-$\gamma^{-/-}$ recipients. To identify the mechanism of the early protection of the liver and spleen, we transferred CFSE-labeled N-WT, M-WT, M-IFN-$\gamma^{-/-}$, or M-Prf$^{-/-}$ CD8$^+$ T cells into double-congenic B6.D2-D6 mice carrying the Thy1.1 allele (B6.D2-D6-Thy1.1). Consistent with our previous experiments in BALB/c mice, there were no significant donor- or host-derived CD8$^+$ T responses in the liver or spleen at 4 dpi (data not shown), indicating that the protection of the liver and spleen at 4 dpi was not due to in situ responses. However, at 4 dpi, the D-LNs of M-WT- and M-IFN-$\gamma^{-/-}$- but not M-Prf$^{-/-}$-recipient mice were enlarged, with more than twice the total number of cells than uninfected mice or N-WT recipients

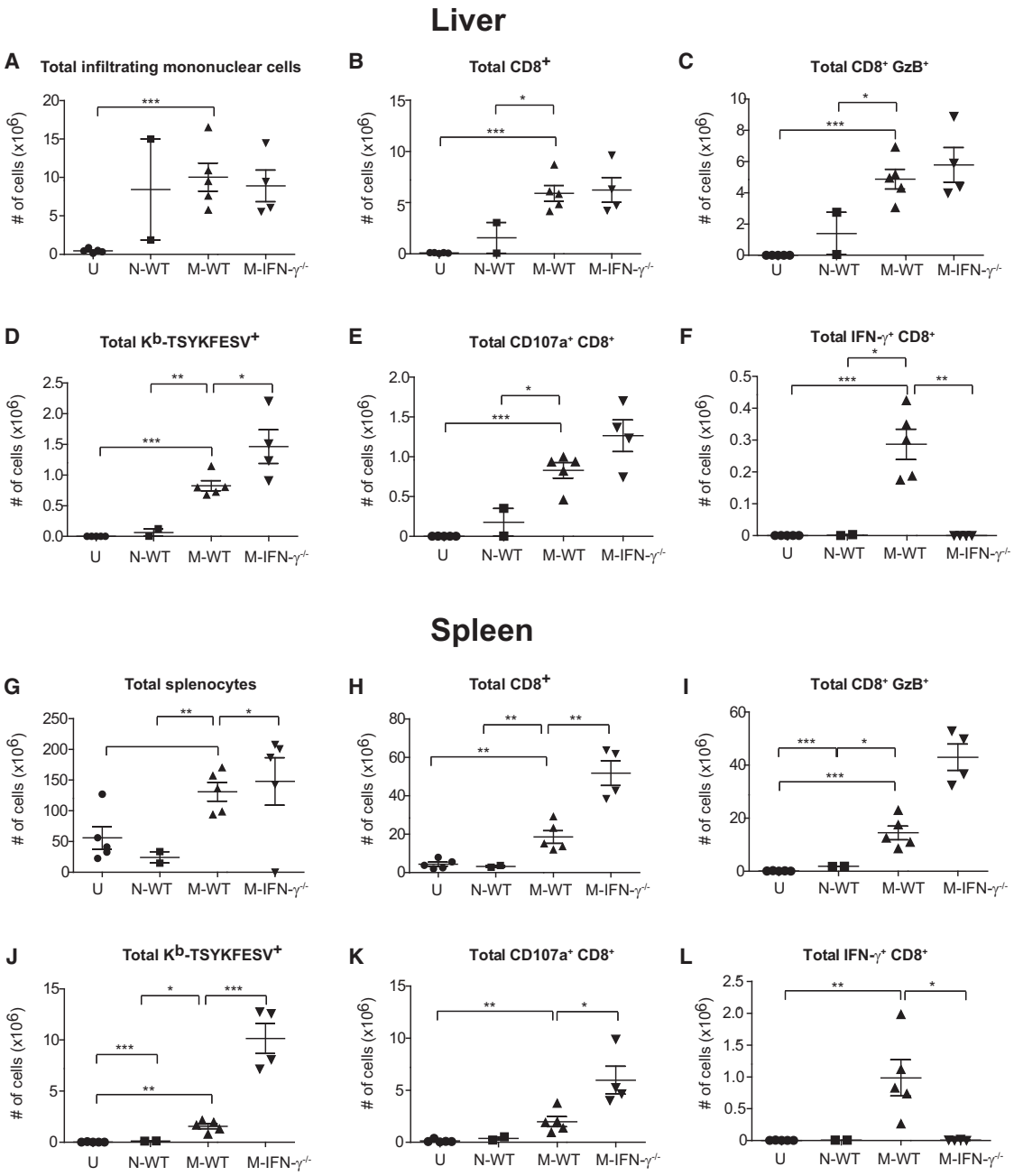



**Figure 2. M-WT and M-IFN-$\gamma^{-/-}$ CD8+ T Cells Respond Strongly in the Liver and Spleen of IFN-$\gamma^{-/-}$ Mice**

IFN-$\gamma^{-/-}$ mice received 5 × $10^6$ N-WT, M-WT, or M-IFN-$\gamma^{-/-}$ CD8+ T cells. One day later, the mice were infected with ECTV, and at 7 dpi the mononuclear cells infiltrating the livers (A–F) and splenocytes (G–L) were incubated for 5 hr with TSYKFESV or without peptide, and the indicated parameters were determined. Data correspond to an experiment with five mice per group ± SEM, with exception of N-WT, which had two mice per group because three mice died at 7 dpi. Data are representative of two experiments. Data are represented as mean ± SEM. See also Figure S2 for representative flow cytometry plots.

(Figure 4C). In addition, compared to uninfected mice, the total numbers of CD8+ T cells in the D-LN of M-WT and M-IFN-$\gamma^{-/-}$ were significantly increased but not different in Prf$^{-/-}$ recipients. On the other hand, the relative (data not shown) and absolute numbers of CD8+ T cells were significantly decreased in N-WT recipients (Figure 4D) The number of donor (Thy1.2+) CD8+ T cells in M-WT and M-IFN-$\gamma^{-/-}$ recipients was also significantly higher than in N-WT or M-Prf$^{-/-}$ recipients (Figures 4E and 4F). Most M-WT and M-IFN-$\gamma^{-/-}$ but not M-Prf$^{-/-}$ or N-WT cells in the D-LN were effectors because they had divided extensively as indicated by CFSE dilution (Figures 4E and 4G) and expressed GzB (Figure 4H). Thus, the early protection of the liver and spleen in M-WT- and M-IFN-$\gamma^{-/-}$-recipient mice was due to a rapid response in the D-LN that curbed LH spread. The data also

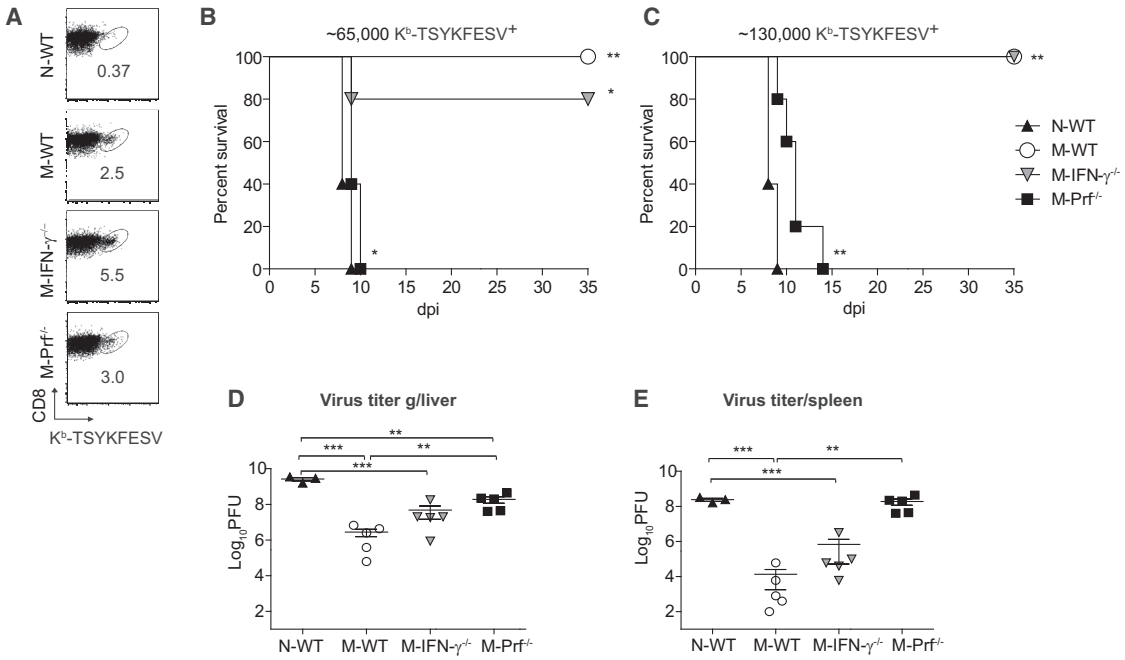



**Figure 3. Memory CD8+ T Cells Deficient in IFN-γ but Not in Prf Protect Susceptible Mice from Lethal Mousepox**

$CD8^+$ cells were magnetically purified from pooled LNs and spleens from donor naive or VACV-immune B6, IFN-$\gamma^{-/-}$, or $Prf^{-/-}$ mice. $CD8^+$-purified $CD8^+$ T cells ($10^6$, $2.5 \times 10^6$ or $5 \times 10^6$) from each type of donor cell were transferred intravenously into groups of five B6.D2-D6 mice. One day later, the mice were infected with ECTV and survival was monitored.

(A) The frequency of $CD8^+$ T cells specific for the immunodominant determinant TSYKFESV was determined by staining with $K^b$-TSYKFESV dimers.

(B) Kaplan-Meier survival curve for mice transferred with ~65,000 $K^b$-TSYKFESV$^+$ cells as determined from the results in (A).

(C) Kaplan-Meier survival curve for mice transferred with ~130,000 $K^b$-TSYKFESV$^+$ cells as determined from the results in (A). All mice transferred with $10^6$ M-WT or M-$Prf^{-/-}$ (~25,000 $K^b$-TSYKFESV$^+$ cells) succumbed to the infection, all the mice transferred with $5 \times 10^6$ M-IFN-$\gamma^{-/-}$ (~270,000 $K^b$-TSYKFESV$^+$ cells) survived and are not displayed graphically.

(D and E) Virus titers at 7 dpi in livers (D) and spleens (E) from B6.D2-D6-Thy1.1$^+$ mice that received $5 \times 10^6$ N-WT $CD8^+$ T cells or enough M-WT, M-IFN-$\gamma^{-/-}$, or M-$Prf^{-/-}$ $CD8^+$ T cells to contain ~75,000 $K^b$-TSYKFESV$^+$ cells. Data are represented as a mean ± SEM and are representative of two independent experiments.

suggest that the inability of M-$Prf^{-/-}$ cells to control the virus (probably due to their failure to rapidly kill infected cells) curtailed their own response in the D-LN (likely because they became infected and died).

We have previously shown that B6.D2-D6 mice infected with WT ECTV do not mount primary $CD8^+$ T cell responses to ECTV and succumb to mousepox (Fang et al., 2011). However, treatment of B6.D2-D6 mice with the antiviral drug Cidofovir at 2 dpi prevented mousepox (data not shown) and enabled a strong primary $CD8^+$ T cell response (Figure S3A). This demonstrates that the failure of B6.D2-D6 mice to generate potent primary $CD8^+$ T cell responses during ECTV infection is not due to intrinsic defects of the $CD8^+$ T cells or accessory cells but, most likely, to excessive viral replication. Thus, we studied whether, similar to Cidofovir, protective memory $CD8^+$ T cells enable primary $CD8^+$ T cell responses to ECTV by looking for a primary anti-ECTV $CD8^+$ T cell response ($Thy1.2^-$) in the D-LN of the B6.D2-D6-Thy1.1-recipient mice. Interestingly, the absolute number of total $Thy1.2^-$ $CD8^+$ T cells (Figure 4I) and of $Thy1.2^-$ $GzB^+$ $CD8^+$ T cells (Figure 4J) was significantly higher in M-WT and M-IFN-$\gamma^{-/-}$ recipients than in N-WT recipients. Thus, protective M-WT and M-IFN-$\gamma^{-/-}$ $CD8^+$ T cells enabled a primary $CD8^+$ T cell response in the D-LN. Given the small size of the D-LNs, a more-detailed analysis of the primary response was not possible in these mice. In a related experiment, naive (N-EGFP) or VACV-immune (M-EGFP) $CD8^+$ T cells from C57BL/6-Tg(CAG-EGFP)1Osb/J (B6-EGFP) mice that express enhanced green fluorescence protein (EGFP) ubiquitously were transferred into B6.D2-D6 mice that were infected or not with ECTV expressing the red fluorescence protein mCherry (ECTV-mCherry). The D-LNs were collected at 5 dpi and analyzed by confocal microscopy. The results suggested that M-EGFP decreased the spread of the infection from the paracortical areas into the medulla of the D-LN, providing some insights into how memory cells could prevent virus spread (Figure S3B).

## Endogenous Prf but Not IFN-γ in Memory $CD8^+$ T Cells Is Required for the Late Control of ECTV in the Liver and Spleen

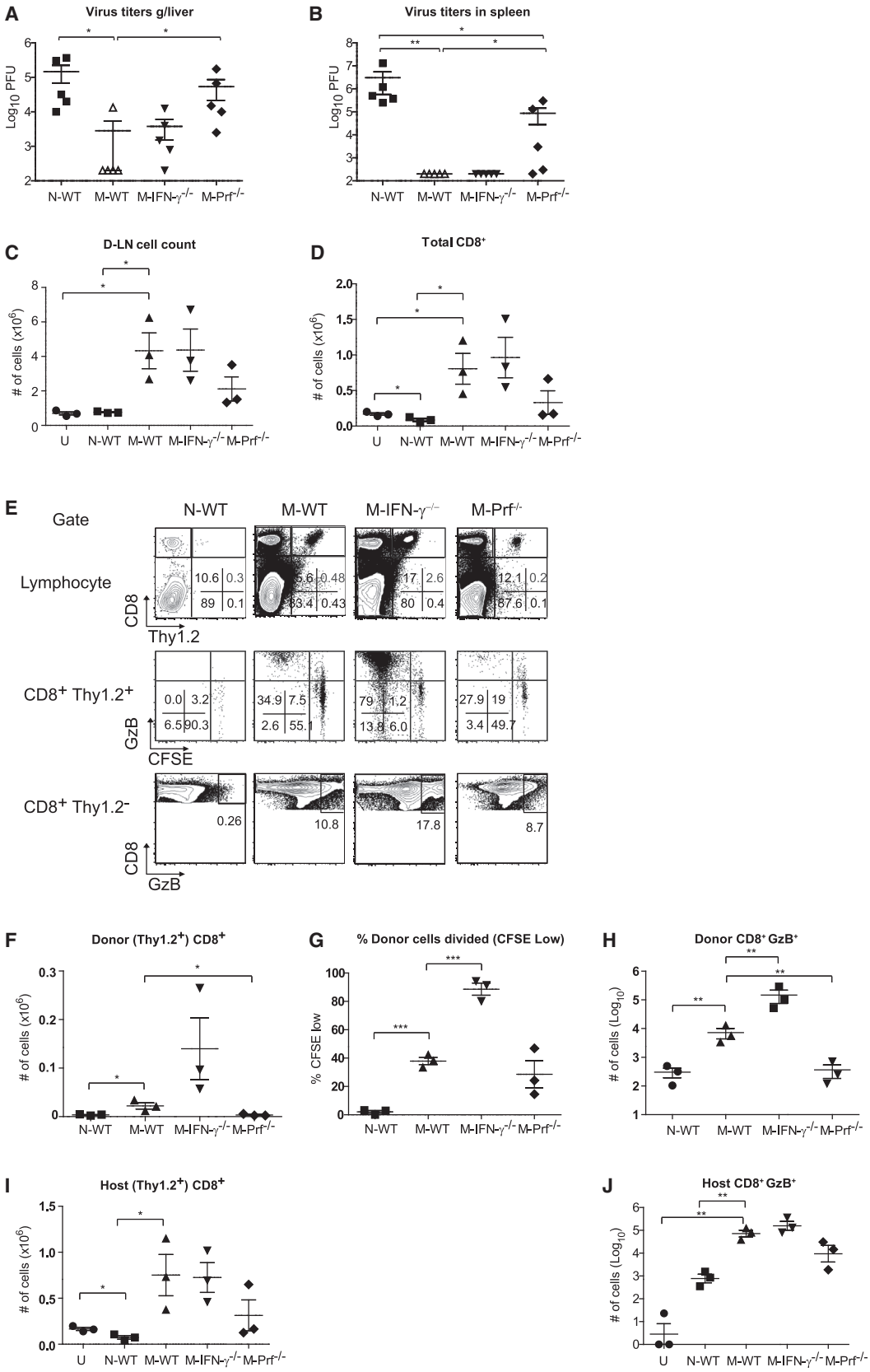
Given that naive B6.D2-D6 mice succumb to ECTV starting at 7 dpi, we also analyzed the response and effects of the different memory $CD8^+$ T cells at this time point in the liver and spleen. M-WT, M-IFN-$\gamma^{-/-}$, and M-$Prf^{-/-}$ recipients had significantly higher numbers of LIMCs as compared to N-WT recipients and uninfected controls (Figure 5A; representative flow cytometry plots for Figure 5 can be found in Figures S4A and S4B). More LIMCs were $CD8^+$ T cells in M-WT and M-IFN-$\gamma^{-/-}$ as compared to M-$Prf^{-/-}$ recipients in relative and absolute numbers (Figures

5B and 5C). Analysis of the donor (Thy1.2+) CD8+ T cell response showed that Kb-TSYKFESV-specific (Figure 5D) and GzB+ LIMCs (Figure 5E) were present in M-WT, M-IFN-γ−/−, and M-Prf−/− recipients but not in N-WT recipients. However, their absolute numbers were significantly higher in M-WT and M-IFN-γ−/− than in M-Prf−/− recipients. After ex vivo restimulation with TSYKFESV, many LIMCs expressed IFN-γ in M-WT and M-Prf−/− recipients but not in M-IFN-γ−/− recipients (Figure 5F). Analysis of the host-derived (Thy1.2−) primary response showed significantly higher numbers of Kb-TSYKFESV-specific (Figure 5G) and GzB+ (Figure 5H) LIMCs in M-WT and M-IFN-γ−/− than in M-Prf−/−. After ex vivo restimulation with TSYKFESV, a significant number of host-derived CD8+ T LIMCs expressed IFN-γ in M-WT, M-Prf−/− and, importantly, also in M-IFN-γ−/− recipients (Figure 5I). Spleens were lymphopenic in most N-WT and M-Prf−/− recipients, while those in M-WT and M-IFN-γ−/− recipients had increased cellularity (Figure 5J). The frequency of splenic CD8+ T cells was unchanged in M-WT and M-IFN-γ−/− recipients, decreased or unchanged in N-WT recipients, and significantly increased in M-Prf−/− recipients, suggesting that activated cells were not killed by the virus but resting cells were (Figure 5K). As compared to uninfected mice, the absolute numbers of CD8+ T cells in the spleens of N-WT recipients were significantly decreased, but they were not significantly affected in recipients of memory CD8+ T cells (Figure 5L). Analysis of Thy1.2+ donor-derived CD8+ T cells showed significantly higher numbers of Kb-TSYKFESV-specific (Figure 5M) and GzB+ (Figure 5N) cells in M-WT, M-IFN-γ−/−, and M-Prf−/− as compared to N-WT recipients. After ex vivo restimulation with TSYKFESV, a significant number of donor-derived CD8+ T cells expressed IFN-γ in M-WT and M-Prf−/− but not in M-IFN-γ−/− recipients (Figure 5O). While smaller compared to the donor-derived, significant numbers of host-derived (Thy1.2−) Kb-TSYKFESV-specific (Figure 5P) and GzB+ (Figure 5Q) cells were present in M-WT, M-IFN-γ−/−, and M-Prf−/− but not in N-WT recipients. After ex vivo restimulation with TSYKFESV, a small but significant number of host-derived CD8+ T cells expressed IFN-γ in M-WT, M-Prf−/−, and also M-IFN-γ−/− but not N-WT recipients (Figure 5R). Thus, M-IFN-γ−/− cells were at least as effective as M-WT cells at mounting a response in the liver and spleen, at enabling a primary response that produced IFN-γ, and at protecting from splenocyte loss. On the other hand, M-Prf−/− CD8+ T cells were less able to respond or enable a primary response in the liver, and while they responded strongly in the spleen, they were unable to protect from splenocyte loss.

It was of interest to determine whether concomitant primary responses to ECTV can also occur in resistant B6 mice that normally mount a strong primary response to ECTV and whether very high numbers of memory CD8+ T cells could inhibit the concomitant primary response. For this purpose, B6 mice were transferred with 5 × 10^6 (M-WT^LOW) or 30 × 10^6 (M-WT^HIGH) CD8+ T cells from VACV-immune mice, and their CD8+ T cell responses were determined in the spleen at 7 dpi (Figure S4C). We found that although they were reduced in comparison to untransferred controls, concomitant primary responses occurred in the presence of both M-WT^HIGH and M-WT^LOW cells. While the presence of concomitant primary and secondary responses have been described for other pathogens infected through the intravenous or respiratory routes (Badovinac et al., 2003; Martin et al., 2011, 2012; Turner et al., 2001), whether they are unique to ECTV or also occur for other pathogens when introduced via the footpad has not been explored. We found concomitant primary and secondary responses not only to VACV (which is nonpathogenic but antigenically almost identical to ECTV) but also to lymphocytic choriomeningitis virus (LCMV) Armstrong, a nonlytic RNA virus that naturally infects mice but does not cause disease after footpad inoculation (Figures S4D and S4E). In the presence of the memory cells, primary responses to VACV were partially inhibited, but those to LCMV were unchanged. Thus, after footpad infection, concomitant primary and secondary responses occur not only to ECTV in mousepox susceptible mice but also to ECTV in mousepox-resistant mice, to nonpathogenic VACV, and to noncytolytic LCMV.

## N-WT T Cells Can Complement M-IFN-γ−/− CD8+ T Cells to Protect IFN-γ−/− Mice from Mousepox

Not only CD8+ T but also CD4+ T and NKCs can produce IFN-γ. We therefore investigated whether any of these populations from naive B6 mice could complement the ability of the IFN-γ−/− memory CD8+ T cells to protect from mousepox. In a preliminary experiment, we found that most IFN-γ−/− mice that received M-IFN-γ−/− CD8+ T cells combined with a large number (4 × 10^7) of total leukocytes pooled from spleens, LNs, and livers of naive mice survived (N-WT leukocytes). Reduction of the dose of N-WT leukocytes resulted in decreased survival with all the mice receiving M-IFN-γ−/− CD8+ T cells combined with only 10^7 N-WT leukocytes succumbing to mousepox. All IFN-γ−/− mice that had been transferred with 4 × 10^7 N-WT leukocytes alone also succumbed to mousepox, indicating that this large number of IFN-γ-sufficient naive cells in the absence of memory CD8+ T cells was not protective. As before, M-IFN-γ−/− cells alone did not protect the IFN-γ−/− mice (Figure S5). Thus, a sufficient number of N-WT leukocytes can complement M-IFN-γ−/− CD8+ T cells to protect from mousepox. To identify which leukocyte populations were capable of complementing the M-IFN-γ−/− CD8+ T cells, we performed adoptive transfer experiments where the donors of N-WT leukocytes had been depleted of NKCs, CD4+ T cells, CD8+ T cells, or CD4+ and CD8+ T cells 1 day before harvest and transfer (Figure 6). All mice that received M-IFN-γ−/− CD8+ T cells combined with total N-WT leukocytes or M-IFN-γ−/− CD8+ T cells combined with N-WT leukocytes from NKC-depleted mice survived the infection without major weight loss. This indicated that production of IFN-γ by NKCs is not essential to complement protection by M-IFN-γ−/− CD8+ T cells. On the other hand, most mice transferred with M-IFN-γ−/− CD8+ T cells together with N-WT leukocytes from mice depleted of CD4+ and CD8+ T cells succumbed to mousepox, indicating that N-WT T cells can complement M-IFN-γ−/− CD8+ T cell protection from mousepox. Most mice that received M-IFN-γ−/− CD8+ T cells together with N-WT leukocytes from either CD4+-T-cell- or CD8+-T-cell-depleted mice survived ECTV challenge, indicating that either CD4+ or CD8+ T cells can complement the protective capacity of M-IFN-γ−/− CD8+ T cells. Control mice that received either M-IFN-γ−/− CD8+ T cells or total N-WT leukocytes alone succumbed to mousepox. Thus, WT CD4+ or CD8+ T cells recruited from the naive pool can complement IFN-γ-deficient memory CD8+ T cells to protect from an acute viral disease.

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## DISCUSSION

Based on correlation, Panchanathan et al. have argued that only antibodies can protect from mousepox (Panchanathan et al., 2010). Contrary to this view, we have previously shown that memory CD8+ T cells are effective at protecting susceptible mice from lethal mousepox and visible symptoms of disease (Remakus et al., 2012; Xu et al., 2007). Furthermore, we have shown that circulating antibodies and memory CD8+ T cells fulfill a similar role; they subdue the spread of the virus, tipping the balance in favor of the host and allowing for the development of a full immune response (Xu et al., 2007). Here we investigated whether intrinsic or extrinsic IFN-γ and granule exocytic killing are required during memory CD8+ T cells protection from mousepox.

Naive IFN-$\gamma^{-/-}$ and IFN-γ-receptor-deficient (IFN-$\gamma R^{-/-}$) mice succumb to footpad ECTV infection within 7–9 dpi (Chaudhri et al., 2004; Karupiah et al., 1993). However, IFN-$\gamma R^{-/-}$ mice resist a secondary challenge with virulent ECTV if previously immunized with an attenuated ECTV strain, indicating that IFN-γ is not essential for resistance to secondary ECTV infection. However, this protection was mediated by antibodies (Panchanathan et al., 2005). Here we have shown that IFN-γ is not essential for strong memory CD8+ T cell responses to ECTV, because M-IFN-$\gamma^{-/-}$ CD8+ T cells responded swiftly in IFN-$\gamma^{-/-}$ mice. Indeed, they responded stronger than M-WT CD8+ T cells most likely because in these experiments we transferred equal number of total CD8+ T cells that have higher frequency of memory cells in VACV-immunized IFN-$\gamma^{-/-}$ than WT B6 mice (Remakus and Sigal, 2011) or, less likely, because a higher rate of expansion of the M-IFN-$\gamma^{-/-}$ cells. Still, despite their strong responses, M-IFN-$\gamma^{-/-}$ CD8+ T cells did not protect M-IFN-$\gamma^{-/-}$ mice from mousepox. This indicates that, distinct from antibody-mediated protection, the presence of IFN-γ is crucial during memory CD8+ T cell protection. On the other hand, M-WT CD8+ T cells fully protected IFN-$\gamma^{-/-}$ mice from mousepox, demonstrating that the responding memory cells can be the sole source of the necessary IFN-γ.

Despite the need for IFN-γ during protection by memory CD8+ T cells, we also found that WT and IFN-$\gamma^{-/-}$ memory CD8+ T cells controlled early ECTV LH spread and protected susceptible B6.D2-D6 mice from mousepox. This was the direct result of their ability to kill infected cells in the D-LN because Prf$^{-/-}$ memory CD8+ T cells did not prevent early LH spread or protect from mousepox. Consequently, memory CD8+ T cells have to be capable of Prf-dependent killing but not IFN-γ production to prevent mousepox in susceptible B6.D2-D6 mice.

We also found that protection by IFN-γ-sufficient and -deficient memory CD8+ T cells was highly dependent on their initial frequency. Hence, while ~25,000 M-WT CD8+ T cells did not protect any mouse from mousepox, ~60,000 or more of these cells was highly protective. Accordingly, the requirement for a high frequency of memory CD8+ T cells may be due to the need for direct contact between infected cells and the memory CD8+ cells to exert killing. Hence, we suggest that it may be necessary to revise how physiologically relevant CD8+ T cell memory is currently measured. While polyfunctionality is a correlate of disease progression in elite HIV controllers, the absence of polyfunctional memory T cells after vaccination (Betts et al., 2006; Kannanganat et al., 2007; Precopio et al., 2007) may not necessarily indicate suboptimal protection. Thus, instead of assessing T cell functional superiority by the ability of T cells to coproduce multiple cytokines, it may be important to focus on memory CD8+ T cell frequency or their ability to protect from disease.

Given that IFN-γ is absolutely required during protection by memory CD8+ T cells, it was important to identify the source of IFN-γ during protection by M-IFN-$\gamma^{-/-}$ cells. After virulent ECTV infection, naive B6 mice mount very strong primary CD8+ T cell responses, while naive B6.D2-D6 mice do not (Fang et al., 2011). Our experiments show that in the presence of memory CD8+ T cells, the primary CD8+ T cell responses of B6.D2-D6 mice to virulent ECTV were enabled in the D-LN, liver, and spleen. Moreover, we showed that protection of IFN-$\gamma^{-/-}$ mice by M-IFN-$\gamma^{-/-}$ could be achieved if adequate numbers of IFN-$\gamma^{+}$ naive T cells were present. While some reports have demonstrated that memory CD8+ T cells can outcompete naive CD8+ T cells by limiting their access to or by killing antigen presenting cells (Guarda et al., 2007; Kedl et al., 2000), our finding of concomitant primary and secondary responses after footpad infection is not unique to B6.D2-D6 mice or to ECTV infection, but also occurs after infection of resistant B6-CD45.1 mice with virulent ECTV even after transfer of very large numbers of memory CD8+ T cells and after infection with nonvirulent VACV and noncytolytic LCMV. Simultaneous primary and secondary responses have also been shown for other pathogens (Badovinac et al., 2003; Martin et al., 2011, 2012; Turner et al., 2001). However, in most of these cases and in our experiments with VACV, there was partial inhibition of the primary response probably because the pathogen is well controlled in the naive host. On the other hand, the primary response to virulent ECTV was enabled.

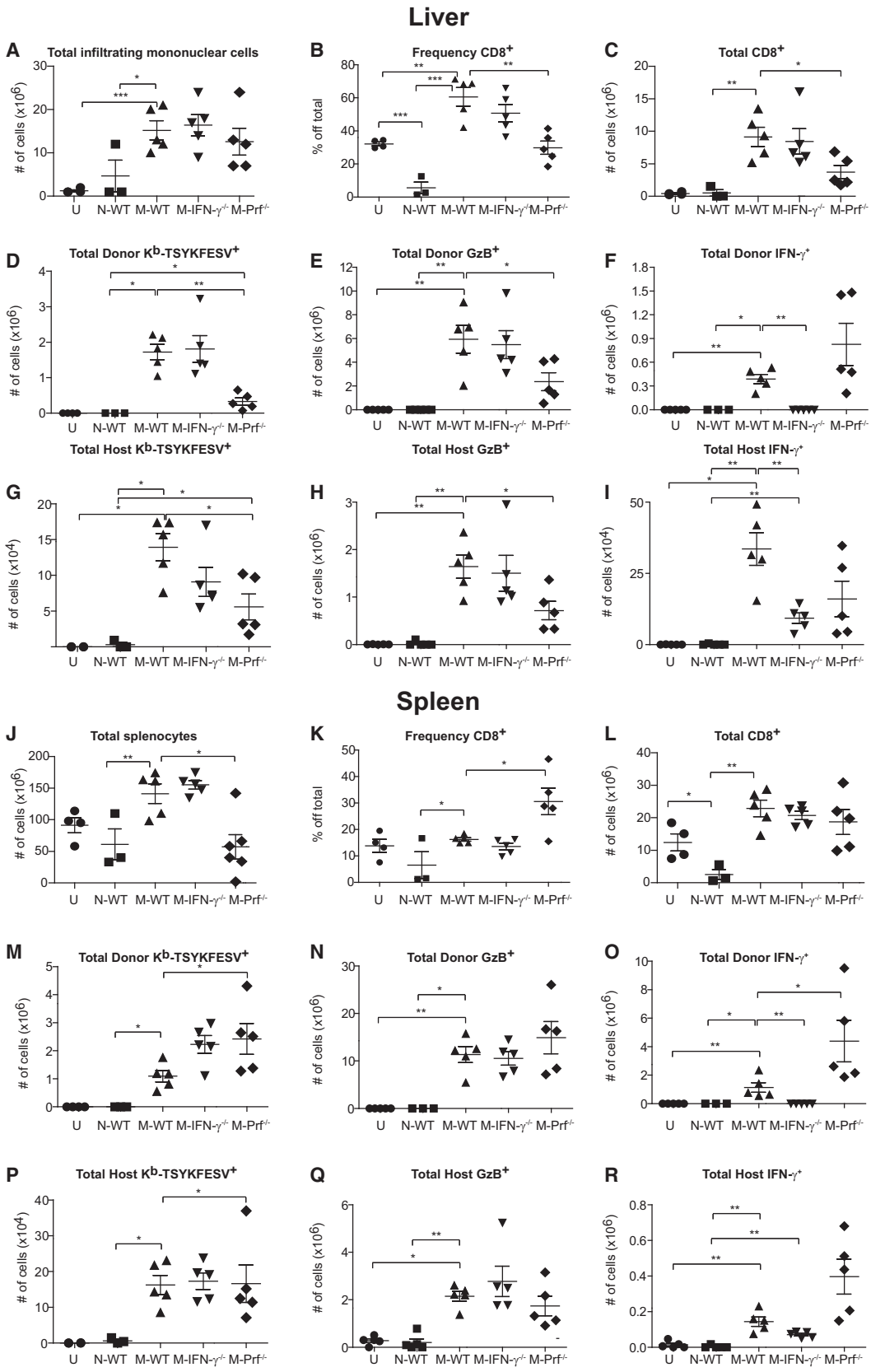
How do the memory CD8+ T cells enable the primary response to virulent ECTV? Several lines of evidence suggest that the inability of naive B6.D2-D6 mice to mount primary CD8+ T cell responses to virulent ECTV is due to the excessive replication of the virus and not to intrinsic deficiencies in the CD8+ T cells or the antigen presenting cells: (1) *Klrd1*$^{-/-}$ mice, which have the exact same genetic deficiency as B6.D2-D6 mice, mount perfectly normal CD8+ T cell responses to VACV, which shares

**Figure 4. Endogenous Prf but Not IFN-γ in Memory CD8+ T Cells Is Required for the Early Control of ECTV LH Spread**

(A and B) Virus titers at 7 dpi in livers (A) and spleens (B) from B6.D2-D6 mice that received 5 × $10^6$ N-WT CD8+ T cells, or enough M-WT, M-IFN-$\gamma^{-/-}$, or M-Prf$^{-/-}$ CD8+ T cells to contain ~75,000 $K^b$-TSYKFESV+ cells. Data are representative of three independent experiments.

(C–J) N-WT, M-WT, M-IFN-$\gamma^{-/-}$, or M-Prf$^{-/-}$ CD8+ T cells were labeled with CFSE to identify divided donor cells. Five million N-WT CD8+ T cells or a number of M-WT, M-IFN-$\gamma^{-/-}$, or M-Prf$^{-/-}$ CD8+ T cells that contained ~75,000 $K^b$-TSYKFESV+ cells were transferred into B6.D2-D6-Thy1.1+ mice. One day later, the mice were infected with ECTV, and at 4 dpi D-LN cells were counted and analyzed by flow cytometry. The indicated parameters were analyzed. Data are represented as a mean ± SEM and are representative of two similar experiments. Representative flow cytometry plots are shown in (E).

See also Figure S3 for Cidofovir-treated mice and confocal microscopy.

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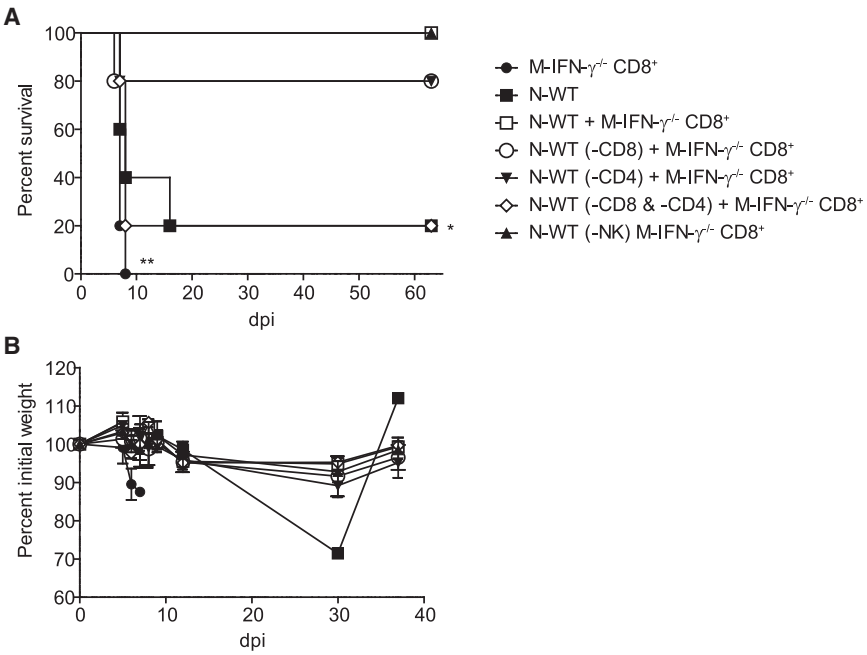


**Figure 6. N-WT T cells can complement M-IFN-$\gamma^{-/-}$ CD8$^+$ T cells to protect IFN-$\gamma^{-/-}$ mice from mousepox.**

IFN-$\gamma^{-/-}$ mice were transferred with 2.5 × $10^6$ M-IFN-$\gamma^{-/-}$ CD8$^+$ T cells and/or with 5 × $10^7$ leukocytes (pooled splenocytes, LN cells, and liver mononuclear cells) from naive B6 mice that had been depleted or not of CD4$^+$, CD8$^+$, CD4$^+$, and CD8$^+$ T cells or NKCs as indicated. One day after transfer, the mice were challenged with ECTV in the footpad. Survival (A) and weight loss (B) are shown. Data correspond to the mean of five mice per group ± SEM and are representative of two independent experiments. See also Figure S5.

the same determinants with ECTV but replicates poorly in mice (Fang et al., 2011). (2) CD8$^+$ T cells from B6.D2-D6 and *Klrd1*$^{-/-}$ mice mount similar responses when adoptively transferred to B6 mice (Fang et al., 2011). (3) Inhibition of viral replication with the antiviral drug Cidofovir at 2 dpi enabled the primary CD8$^+$ T cell response to virulent ECTV in B6.D2-D6 mice (Figure S3A). Hence, the enablement of a concomitant naive response to virulent ECTV by the memory CD8$^+$ T cells resulted from their ability to reduce virus replication. This reduction in virus replication was mostly the consequence of cytolytic killing, because Prf$^{-/-}$ M-CD8$^+$ T cells did not reduce virus replication or efficiently enable a primary response.

In summary, our experiments demonstrate that during protection by memory CD8$^+$ T cell from a lethal OPV disease that spreads LHY, IFN-γ and cytolytic killing are both essential. However, while the production of IFN-γ can be outsourced to concomitant primary effectors, the ability of the responding memory CD8$^+$ T cells to kill infected cells cannot be replaced. These data are important for our understanding of antiviral immunity and should be instrumental for designing and evaluating antiviral vaccines.

## EXPERIMENTAL PROCEDURES

### Viruses

Stocks were produced and titers determined as previously described (Fang et al., 2011; Fang and Sigal, 2006, 2010; Xu et al., 2007).

### Mice and Infections

The Fox Chase Cancer Center Institutional Animal Care and Use Committee approved the experimental protocols involving animals. C57BL/6 (B6) mice were purchased from Taconic when they were 8–10 weeks of age and rested at least 1 week before use in experiments. The B6.D2-D6, IFN-$\gamma^{-/-}$, and B6-EGFP mice were initially purchased from Jackson Laboratory and were bred in the Fox Chase Cancer Center Laboratory Animal Facility. B6.D2-D6 Thy1.1 mice were generated by crossing of B6.D2-D6 with B6.PL-Thy1a/CyJ mice (Jackson) and genotyped by staining of peripheral blood mononuclear cells for Thy1.1$^+$, NK1.1$^-$, and Ly49H. Unless otherwise indicated, mice were infected with ECTV in the left footpad with 30 μl PBS containing 3 × $10^3$ plaque-forming units (pfu). VACV was inoculated via the intraperitoneal route with 500 μl PBS containing $10^6$ pfu and boosted similarly 4 weeks later. After infections, mice were observed daily for signs of disease (lethargy, ruffled hair, weight loss, skin rash, and eye secretions) and imminent death (unresponsiveness to touch and lack of voluntary movements). When required, mice were treated with 400 μg Cidofovir as previously (Fang and Sigal, 2010).

### Histopathology

Histopathology was as previously described (Xu et al., 2012).

### Adoptive Transfers

CD8$^+$ cells were magnetically purified from LNs and spleens with an Automacs magnetic cell sorter (Miltenyi Biotechnology) at the normal setting as previously described (Fang and Sigal, 2006; Xu et al., 2007). The efficiency of the purification and percent of virus-specific cells was monitored by K$^b$-TSYKFESV Dimer-X staining (BD PharMingen) and fluorescence-activated cell sorting analysis. Unless otherwise indicated, cells were resuspended in PBS ($10^7$/ml) and 500 μl was inoculated intravenously into recipient mice. For experiments in Figure 4, donor cells were labeled with 4 μm CFSE (Invitrogen) as done previously (Xu et al., 2007). For the experiments in Figures 6 and S5, M-IFN-$\gamma^{-/-}$ cells were obtained as above, and the complementing leukocytes were obtained from the LNs, spleen, and livers of naive B6 mice. When indicated, the B6 donor mice had been depleted 24 hr before transfer with 100 μg depleting CD4 (GK1.5, ATCC #HB-191), 100 μg CD8 (2.43), or 200 μg NK (PK136) monoclonal antibodies in 0.5 ml PBS intraperitoneally. Depletion was assessed by flow cytometry. The depleting monoclonal

**Figure 5. Endogenous Prf but Not IFN-γ in Memory CD8$^+$ T Cells Is Required for the Late Control of ECTV in the Liver and Spleen**

B6.D2-D6-Thy1.1$^+$ mice received 5 × $10^6$ N-WT CD8$^+$ T cells or enough M-WT, M-IFN-$\gamma^{-/-}$, or M-Prf$^{-/-}$ CD8$^+$ T cells to contain ~75,000 K$^b$-TSYKFESV$^+$ cells. One day later, the mice were infected with ECTV, and at 7 dpi liver-infiltrating mononuclear cells (A–I) and splenocytes (J–R) were incubated for 5 hr with TSYKFESV or without peptide, and the indicated parameters were determined. Data correspond to five mice per group, with exception of uninfected mice, which had four mice per group, and N-WT, which had three mice per group, because two mice died at 7 dpi. Data are represented as a mean ± SEM and are representative of two experiments with B6.D2-D6 Thy1.1$^+$ recipients (shown) and a third experiment using B6.D2-D6 mice as recipients. See also Figure S4 for representative flow cytometry plots and for higher ECTV doses and VACV and LCMV infection.

antibodies were produced by the FCCC Tissue Culture Facility in CELLine bioreactors (BD) in Cell Animal Free Medium (BD) as directed by the manufacturer. Before adoptive transfer, effective depletion was confirmed by flow cytometry with an aliquot of the leukocytes.

### Flow Cytometry

Flow cytometry was performed as previously described (Fang and Sigal, 2006, 2010; Xu et al., 2007). The following antibodies were used: anti-CD3 (145-2C11, Biolegend), anti-CD4 (GK1.5, Biolegend), anti-CD8a (53-6.7, Biolegend), anti-Thy1.1 (OX-7, Biolegend), anti-Thy1.2 (30-H12, Biolegend), anti-IFN-γ (clone XMG1.2, Biolegend), anti-CD14 (Sa14-2, Biolegend), anti-CD16 (93, Biolegend), anti-CD19 (6D5, Biolegend), anti-CD94 (18d3, Biolegend), anti-CD49b (DX5, BD), anti-CD107a (1D4B, Biolegend), anti-NK1.1 (PK136, BD), anti-Ly49C/F/I/H (14B11, BD), anti-NKp46 (29A1.4, eBioscience), and PECy5.5-labeled anti-human GzB (GzB, Caltag) that cross-reacts with mouse GzB (Wolint et al., 2004). The hybridoma producing monoclonal antibody 25-D1.16 was grown in Cell Line Bioreactors (BD) in animal-protein-free media as recommended by the manufacturer. The 25-D1.16 monoclonal antibody in the supernatant was purified by ammonium sulfate precipitation via standard techniques and labeled with an APEX Alexa 647 Antibody Labeling Kit (Invitrogen) as recommended by the manufacturer. For TSYKFESV-specific TCD8+, H-2K$^b$:Ig recombinant fusion protein (Dimer-X, BD) was incubated with synthetic TSYKFESV (GenScript) and used as recommended by the manufacturer. Monoclonal antibody 25.D-1-stained cells were analyzed by flow cytometry at the Fox Chase Cell Sorting Facility with a LSR II system (BD). At least 100,000 cells were analyzed.

### Data Display and Statistical Analysis

Unless otherwise indicated, all displayed data correspond to one representative experiment of at least two similar experiments with groups of three to six mice. Statistical analysis was performed with Graph Pad Prism software. For survival studies, p values were obtained with the log-rank (Mantel-Cox) test. All other statistical analyses were performed with an unpaired two-tailed t test or the Mann-Whitney test as applicable. When applicable, data is displayed with mean ± SEM. p values were determined between M-WT recipients and all other groups. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. When not marked, the differences were not statistically significant. Unless specifically indicated, all groups were compared to M-WT recipients.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2013.04.004.

## ACKNOWLEDGMENTS

We thank Fox Chase Cancer Center (FCCC) Laboratory Animal, Flow Cytometry, Imaging, Histopathology, and Tissue Culture Facilities, Ms. Holly Gillin for assistance in the preparation of the manuscript, Dr. Andres Klein-Szanto for histopathology, Dr. E. John Wherry (University of Pennsylvania) for GP33 tetramers, Dr. Glenn Rall for LCMV, and Dr. Laurence Eisenlohr for critical reading of the manuscript. This work was supported by grants R01AI048849, R01AI065544, and 5U19AI083008 to L.J.S. and P30CA006927 to the FCCC. S.R. was partially supported by NIH grant T32 CA-009035036 to the FCCC.

Received: June 29, 2011
Revised: February 15, 2013
Accepted: March 27, 2013
Published: May 15, 2013

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